Fast Analysis of Fat-Soluble Vitamins in Infant Milk Powder by Heart Cutting 2D-LC

Application Note

Food Testing and Agriculture

Authors

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Abstract

We developed a heart cutting 2D-LC method for the fast analysis of fat-soluble vitamin A, vitamin E, and vitamin D3 in infant formula milk powder. After saponification, extraction, and filtration, the milk powder sample was injected directly onto the heart cutting 2D-LC system for analysis. The results for vitamin A (VA) and vitamin E (VE) could be obtained directly after the first-dimension separation. After preliminary purification using the first dimension, vitamin D (VD) was then captured by a column cartridge, followed by separation and detection in the second dimension. The entire analytical process lasted only for 14 minutes, and the VA, VE, and VD content was obtained at the same time. The method demonstrated excellent linearity. For VD3, a limit of detection (LOD) of 10 µg/kg (or 400 IU/kg) was reached. The method also showed good reproducibility. For three consecutive injections, RSD of retention time was less than 0.3%, and the RSD of peak area was less than 1% for VA, VE, and VD.

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Introduction

Infant formula milk powder is also known as humanized milk powder. To meet the nutritional needs of infants, various nutrients must be added, resulting in a very complex composition. Fat-soluble vitamins, including vitamins A, D, and E are frequently used as additives. The amounts of VA and VE are relatively large and easy to determine. However, the amount of VD is relatively small and much more difficult to determine due to weak UV absorption and serious matrix interference. In one method described in the literature [1], the sample was purified and concentrated by a solid phase extraction cartridge after saponification, and was then separated and detected by LC/MS. According to the Chinese National Standard [2], the saponified solution must be purified by normal-phase LC to eliminate interferences from the sample matrix, before the determination of VD. The entire process is very cumbersome and time-consuming, resulting in a low sample throughput.

We employed a heart-cutting method to analyze vitamins A, D, and E. VD was purified by the first-dimension column while analyzing VA and VE, and was then introduced into the second-dimension column for separation and determination. Only one sample injection was needed during the whole analytical process for determination of VA, VE, and trace VD. This method not only saves much preparation work, but also provides good stability, and is, therefore, suitable for conventional rapid determination of fat-soluble vitamins in formula milk powder.

Experimental

Instrument configuration

First dimension

- Agilent 1260 Infinity Quaternary Pump, G1311B
- Agilent 1260 Infinity Autosampler, G1329B
- Agilent 1260 Infinity Thermostatted Column Compartment, G1316A, with built-in 2-position/6-port valve
- Agilent 1260 Infinity Diode Array Detector, G4212B, equipped with 10-mm standard flow cell

Second dimension

- Agilent 1260 Infinity Degasser, G1322A
- Agilent 1260 Infinity Binary Pump, G1312B
- Agilent 1260 Infinity Diode Array Detector, G4212B, equipped with 60-mm ultrasensitive flow cell

The software was Agilent OpenLab CDS ChemStation Edition C01.05[35].

HPLC method

The entire HPLC method used heart-cutting 2D-LC, with three phases. The first phase was the sample injection step and the determination of VA, the valve was in position 1-2. In the second phase, VD was captured online in a column cartridge right after it was eluted from the first column, with the valve switched to position 1-6. In the third phase, with the valve switched back to position 1-2, the first column continued to analyze vitamin E while the second dimension was undergoing the separation and detection of VD. The specific details are shown in Figure 1. In this method, a high-efficiency short column (Agilent ZORBAX RRHT Extend-C18, 4.6 × 50 mm, 1.8 µm) was used in the first dimension for rapid analysis of VA and VE. An Agilent ZORBAX Eclipse PAH, 2.1 × 100 mm, 3.5 µm column in the second dimension improved the separation of VD2 and VD3. Meanwhile, the second-dimension DAD detector was equipped with an ultrasensitive flow cell (60-mm path length) to improve sensitivity. For other details, see Table 1.

Table 1. Heart-cutting parameters.

	First dimension	Second dimension
Flow rate:	1 mL/min	0.6 mL/min
Mobile phase A:	H_2O	ACN
Mobile phase B:	MeOH	MeOH
Autosampler:	10 µL	
Column:	Agilent ZORBAX RRHT Extend-C18, 4.6 × 50 mm, 1.8 µm (p/n 722975-902)	Agilent ZORBAX Eclipse PAH, 2.1 × 100 mm, 3.5 µm (p/n 959793-918)
Capture column:	Agilent ZORBAX Extend-C18, 4.6 × 12.5 mm, 5 µm (p/n 820950-930)	
Column temperature:	35 °C	35 °C
Detector:	0 to 3.5 minutes: 325 nm (4 nm, Ref off) 3.5 to 14 minutes: 290 nm (4 nm, Ref off), 20 Hz	265 nm (4 nm, Ref off), 20 Hz
Valve:	0 to 4.4 minutes: position 1-2 (phase 1) 4.4 to 4.6 minutes: position 1-6 (phase 2) 4.6 to 10 minutes: position 1-2 (phase 3)	

Sample preparation

The sample preparation method was in accordance with Chinese National Standard GB 5413.9—2010, as follows:

1. Weigh 10 g of uniformly mixed milk powder (accurate to 0.1 mg) into a 250 mL flask.
2. Add approximately 50 mL water (45 to 50 °C) to dissolve the milk powder and mix well.
3. Add approximately 100 mL of vitamin C solution (15 g vitamin C in 1 L ethanol). Mix the solution well and add 25 mL of aqueous potassium hydroxide (250 g solid KOH in 200 mL water).
4. Mix well, add a magnetic stirrer bar, charge nitrogen into the solution to expel the air, and then seal the flask with a stopper.
5. Add approximately 300 mL water into a 1,000 mL beaker.
6. Place the beaker on the thermostatic magnetic stirrer. When the water temperature has reached 53 °C ± 2 °C, place the flask into the beaker.
7. After stirring and saponification for about 45 minutes, remove the flask and cool it to room temperature immediately.
8. Use a small amount of water to transfer the saponification liquid into a 500 mL separating funnel.
9. Add 100 mL petroleum ether and gently shake.
10. After the air is expelled, cover with the stopper and shake the funnel at room temperature for about 10 minutes. Then allow the funnel to stand for stratification to occur.
11. Transfer the aqueous phase to another 500 mL separating funnel and conduct the second extraction, as before.
12. Combine with ether and neutralize with water to stop the basic saponified solution from entering the column and to prevent degradation of VE.
13. Filter and dehydrate the ether with anhydrous sodium sulfate.
14. Collect the filtrate into a 500 mL round-bottomed flask and evaporate to near dryness on a rotary evaporator at 40 °C ± 2 °C under nitrogen (never evaporate to dryness).
15. Use 9 mL methanol to transfer the residue into a 10 mL volumetric flask. Bring the residue to a constant volume with methanol and shake to mix well. Pass the solution through a 0.22-µm filter and it is ready for testing.

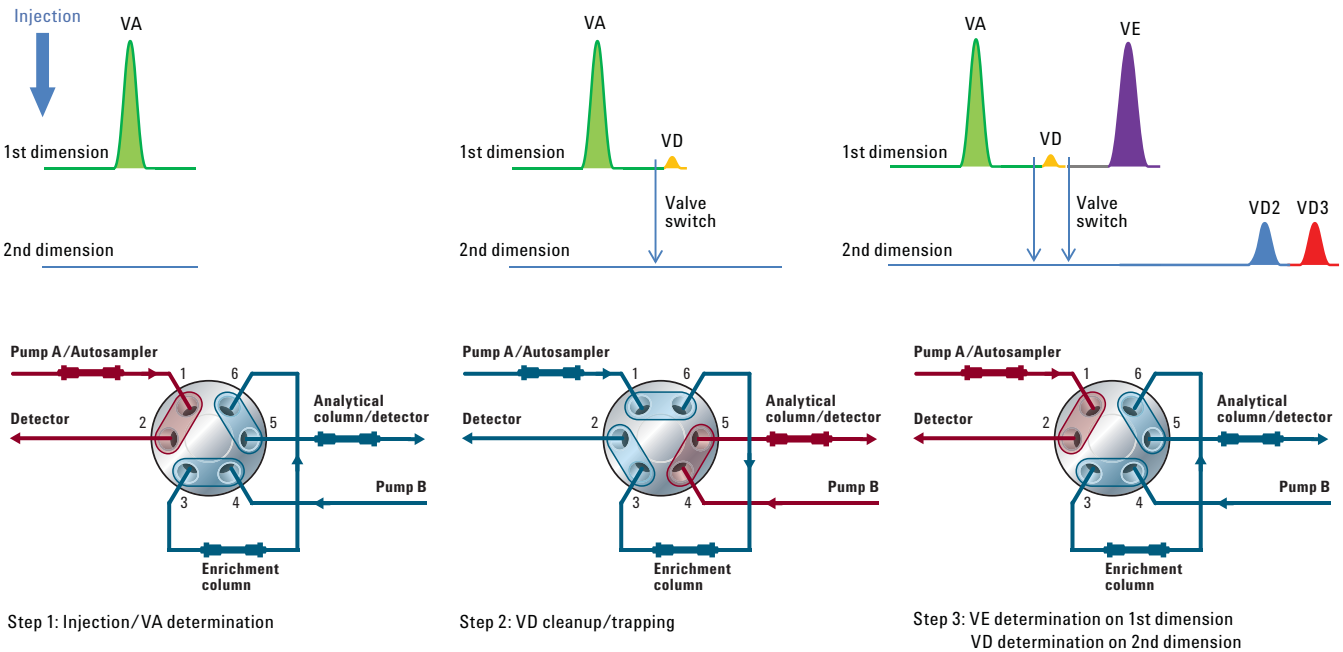

Figure 1. Simultaneous analysis of vitamins A, E, and D2/D3 by heart-cutting 2D-LC.

Results and Discussion

The first-dimension method

VA, VD2 (or VD3), and VE can be easily separated by the reversed-phase C18 column. However, even after the milk powder sample undergoes saponification, and extraction, there will still be some interfering substances. Therefore, the first-dimension gradient should be optimized according to the actual saponified sample to eliminate interference when generating VA and VE peaks. For VD2/VD3, the retention time window is critical. It must be less than 0.2 minutes from the start point of the VD2 peak to the end point of the VD3 peak at a flow rate of 1 mL/mL. Otherwise, the VD2/VD3 would not be captured sufficiently for high recovery. The retention time window was not important for the resolution of VD2 and VD3; even coelution could be acceptable.

Determination of valve timing

Valve timing is critical for the heart cutting method. To improve recovery and purification of VD, the valve timing should be set right before the start point of the VD2 peak and after the end point of the VD3 peak. Taking into account column reproducibility between different batches and the decrease of column efficiency after long-term use, a mixed standard containing VD2 and VD3 should be analyzed with the first-dimension gradient method (Table 1) to redetermine the valve timing. This procedure should be done every time before using the heart cutting method.

The second-dimension method

The second-dimension method was mainly aimed at separating VD2 and VD3, and eliminating interferences from the sample. We discovered that a very simple isocratic elution by ACN/MeOH could achieve the baseline separation of VD2 and VD3 without any interference from the sample matrix on the ZORBAX Eclipse PAH column.

Linearity, limit of detection (LOD), and reproducibility

Both VA and VE contents in infant formula milk powder are relatively high and stable. Therefore, these contents can be accurately measured with qualified sample preparation. For VD, due to its lower content and interferences in the sample, it is necessary to focus on the LOD, linear range, and specificity simultaneously. Figure 2 shows the linear range of VA, VE, and VD3 using our method. The linearity correlation of VA between 2.5 and 12.5 µg/mL was 0.99996, of VE between 50 and 250 µg/mL was 0.99984, and of VD3 between 0.05 and 0.25 µg/mL was 0.99988. Taking signal-to-noise (S/N) = 3, the LOD of VD3 was calculated as 0.01 µg/mL, which was converted to a VD3 content of 10 µg/kg (or 400 IU/kg) in the milk powder sample.

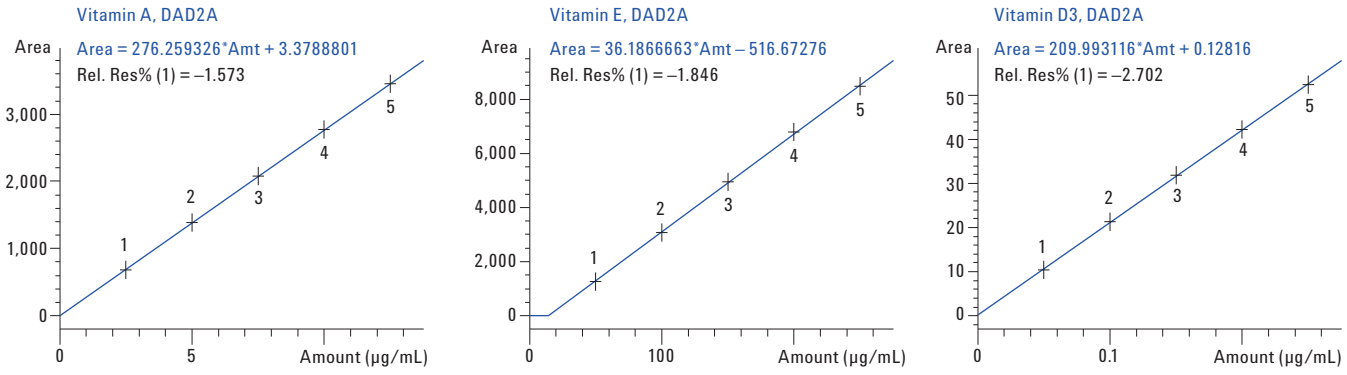

Figure 2. Calibration curves and linear fitting correlation coefficients of vitamins A, E, and D3. Correlation coefficient = 0.99996.

Determination of VD content in milk powder with the method can satisfactorily exclude interference of impurities in the sample matrix and provide high specificity. Figure 3 is the chromatogram of a typical standard and the sample. The actual sample was used to examine the reproducibility of the method. For three consecutive injections, the RSD of retention time was less than 0.3% and the RSD of the peak area was less than 0.81%, as shown in Table 2.

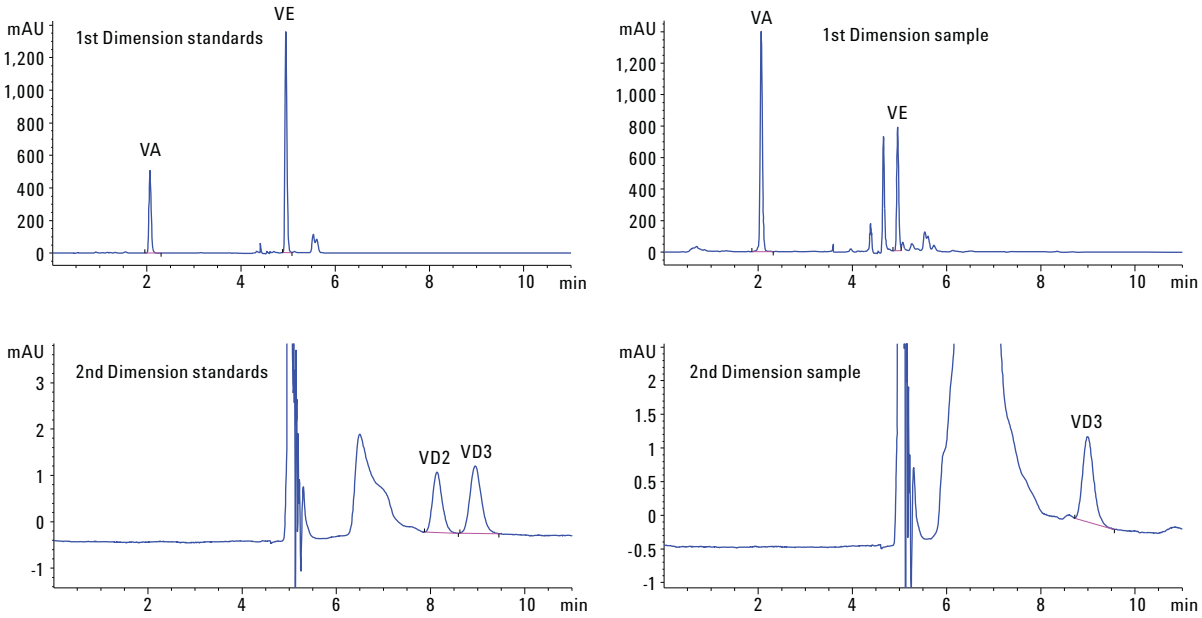

Figure 3. Chromatogram of standards and the sample.

Table 2. Reproducibility of retention time and peak area for three consecutive injections of infant formula.

	Vitamin A		Vitamin E		Vitamin D	
Injection	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area
1	2.064	4645.55273	4.951	2301.32178	8.953	20.24444
2	2.063	4658.86182	4.961	2301.61694	8.991	20.56408
3	2.064	4660.52637	4.965	2301.80396	9.005	20.48096
RSD%	0.03	0.18	0.14	0.01	0.30	0.81

Conclusions

Any Chinese enterprise producing infant formula milk powder should implement inspection of all compounds following the Chinese Regulations on the Supervision and Management on Quality Safety of Milk Products. For the vitamins included in this regulation, national standards specify methods involving HPLC for analysis, and provide a method that gives precedence for confirmation of noncompliance. Such enterprises should, therefore, invest in HPLC. However, to increase productivity and reduce the cost of routine testing, it is more efficient to configure such systems using our method, because it determines VA, VE, and trace VD simultaneously, and because less sample purification is required.

Compared to existing methods for determination of fat-soluble VA, VE, and VD in infant formula milk powder, the heart cutting method described here provides a high degree of automation, easy operation, and low cost. For detection of trace VD, this method is highly sensitive and specific and eliminates the interference of impurities in the sample.

References

1. Heudi, O. *et al. J. Chrom. A* **2004**, *1022*, pp 115-123.
2. Anon. GB 5413.9—2010 Determination of Vitamin A, D, E in Foods for Infants and Young Children, Milk and Milk Products. Chinese National Standards, Beijing.

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